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Selective Translation Initiation on Bicistronic Simian Virus 40 Late mRNA

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We described previously a simian virus 40 (SV40) mutant, pSVAdL, that was defective in synthesis of the late viral protein VP1. This mutant, which contains a 100-base-pair fragment of adenovirus DNA encompassing the major late promoter inserted in the SV40 late promoter region (SV40 nucleotide 294), efficiently synthesizes agnoprotein, a protein encoded by the leader region of the same mRNA that encodes VP1. When the agnoprotein AUG initiation codon in pSVAdL was mutated to UUG, agnoprotein synthesis was abolished, and VP1 synthesis was elevated to wild-type levels. Because levels of late mRNA synthesis were not affected by this mutation, these results support a scanning model of translation initiation and suggest that internal translational reinitiation does not occur efficiently in this situation.

Our understanding of the mechanism of translation initiation in eucaryotic cells has increased considerably in recent years. The initiation of protein synthesis is thought to involve a scanning mechanism in which the 40S subunit of the ribosome attaches to the 5′ end of the mRNA and moves toward the 3′ end until it finds the proper AUG initiation codon (16). At this point, an initiation complex is formed, the 60S ribosomal subunit attaches to the complex, and translation proceeds. Since the vast majority (more than 90%) of eucaryotic mRNAs are monocistronic and lack AUG codons in the 5′ untranslated region (17), the model suggests that in most cases the 40S subunit begins translation at the first AUG sequence it encounters.

In the unusual cases of bicistronic mRNAs or monocistronic messages that contain additional AUG codons in the 5′ untranslated region, the process of selecting the proper AUG initiation codon must be more complicated. Recent work has shown that the sequence immediately surrounding the AUG codon is important for maximal initiation to occur at this site (15, 17, 19). If this sequence is suboptimal, the 40S subunit can bypass the AUG codon and initiate further downstream (19). Consistent with a scanning model and unlike the case with procaryotic translation initiation, the 40S subunit does not appear able to bind to internal AUG codons without first bypassing the more 5′ AUG codons. Ribosomes will not bind to circular RNAs (13, 14), and strong stem-loop structures upstream of the AUG initation codon can inhibit translation (20). A consensus sequence (CCPuCCAUGG) has been derived by examining the sequences around AUG codons utilized for optimal translation initiation (17). Mutagenesis experiments have shown that the purine at position −3 relative to the AUG sequence is most important in conferring the ability to initiate at a particular AUG codon (19). In addition, it has been shown that reinitiation of translation can occur if a termination codon in the frame of an upstream AUG codon occurs 5′ to a second AUG codon (6, 11, 18, 22, 26, 27).

The simian virus 40 (SV40) late 16S message is an example of a bicistronic mRNA (9). This message, which encodes the major capsid protein VP1, also includes an open reading frame in the 5′ leader region that encodes a small protein of unknown function, the agnoprotein (5, 7, 12). It is not completely understood how both VP1 and the agnoprotein can be produced efficiently from this mRNA. The model described above suggests that inefficient use of the agnoprotein AUG initiation codon and possible reinitiation of translation after synthesis of the agnoprotein account for the ability of the mRNA to produce both proteins. However, the sequence around the agnoprotein AUG codon conforms closely to the consensus sequence, which suggests that translation initiation at this site would not be inefficient. In fact, the rate of synthesis of the agnoprotein appears to be very rapid late in the lytic cycle (12). It has been speculated that the agnoprotein itself autoregulates its translation by stabilizing an mRNA conformation that enhances VP1 translation while inhibiting initiation of agnoprotein synthesis (9). However, this hypothesis requires ribosomes to have the ability to bypass the 5′ end of the mRNA and to bind internally at the VP1 initiation codon, which, as discussed above, appears unlikely.

Previously, we constructed mutants of SV40 containing adenovirus type 2 major late promoter (Ad2MLP) sequences from nucleotides −66 to +33 (relative to the adenovirus late RNA initiation site) inserted in the SV40 late promoter region (8). pSVAdL, in which this insert directs transcription towards the late region of SV40, was found to produce extremely low levels of VP1 compared with both wild-type SV40 and a mutant containing the insert in the opposite orientation (pSVAdE), although production of late 16S mRNA was not significantly decreased (8). The majority of the SV40 late RNA initiated from the Ad2MLP, however, and we speculated that the lack of VP1 was caused by the presence of 33 nucleotides of the adenovirus type 2 late leader on 16S mRNA and led to preferential utilization of the agnoprotein AUG codon. Here we show that agnoprotein is indeed produced in pSVAdL-transfected cells, although its synthesis appears not to be increased relative to that in the wild type. However, mutation of the agnoprotein initiation codon to UUG (28) in pSVAdL restores VP1 synthesis to wild-type levels.

To determine whether the agnoprotein was produced by pSVAdL, BSC-1 cells were transfected with linear viral DNA from pSV3 (wild type), pSVAdE, and pSVAdL (Fig. 1). These and all transfections were repeated two or more times with different preparations of DNA. Proteins were labeled at 48 h posttransfection with [^{14}C]leucine for 3.5 h.

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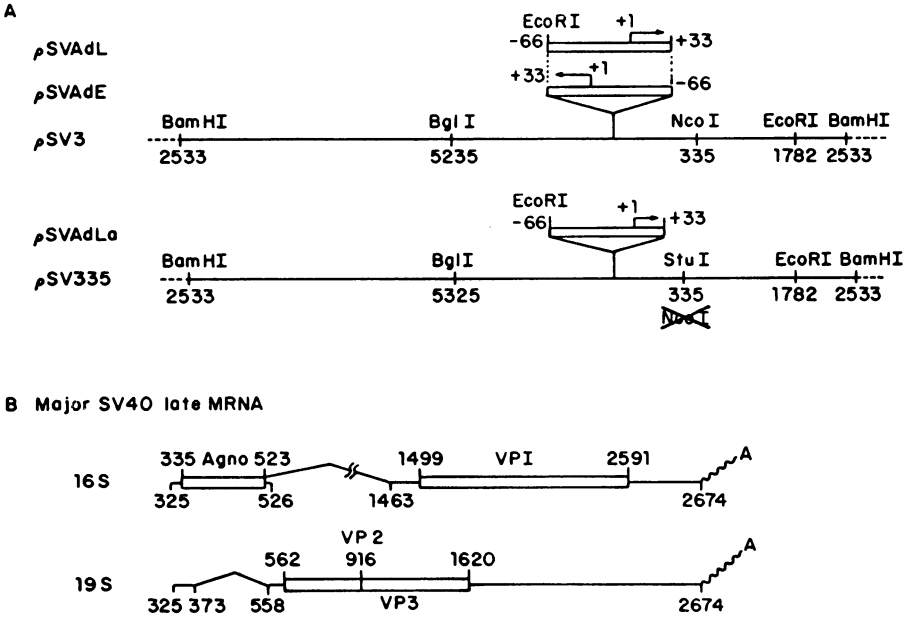

FIG. 1. (A) Structure of recombinant plasmids. The *Nco*I restriction site of pSV335 was changed to an *Stu*I restriction site by an A-to-T transversion (28). The Ad2MLP was inserted into pSV335 as described previously (8). (B) Major late mRNAs. ▭, Coding regions; ⌐¬, introns. Numbers above line designate coding boundaries; numbers below line designate RNA landmarks.

Cells were then harvested, and proteins were isolated and immunoprecipitated with antiagnoprotein antibody (25). When the immunoprecipitated proteins were resolved on a 20% polyacrylamide gel (21), the results (Fig. 2) indicated that agnoprotein was synthesized with equal efficiency in all cases, showing that the 16S mRNA from cells transfected with pSVAdL could be translated as efficiently as wild-type SV40 late mRNA. It thus appears that the Ad2MLP can effect differential translation of agnoprotein and VP1, possibly because of the small segment of the adenovirus type 2 major late leader sequence in the 5′ leader region of much of the late SV40 mRNA.

To determine whether translation of the agnogene in pSVAdL was the cause of reduced VP1 synthesis, the Ad2MLP was inserted into the *Kpn*I site of pSV335 (28). This plasmid (Fig. 1) contains the entire SV40 genome with an A-to-T transversion at SV40 nucleotide 335, which eliminates the initiation codon for the agnoprotein. Viral sequences from this plasmid, pSVAdLa, were transfected into BSC-1 cells, as were viral sequences from pSVRI (wild type), pSVAdE, and pSVAdL. Proteins were labeled at 48 h posttransfection with ^{35}S for 2 h, cells were lysed, and VP1 was immunoprecipitated with anti-SV40 capsid antisera (8). The results of separating these proteins on a 12.5% polyacrylamide gel (Fig. 3) show that, as seen previously, pSVAdL produced a greatly reduced amount of VP1, whereas pSVAdLa produced VP1 at a level essentially identical to that of wild type. Thus, mutation of the AUG initiation codon of the agnoprotein in pSVAdL results in significantly increased levels of VP1 synthesis.

To rule out the possibility that transcription from the Ad2MLP was affected by the mutation in pSVAdLa, nuclease S1 analysis of cytoplasmic RNA extracted at 48 h posttransfection was performed with a single-stranded probe labeled at the *Bst*NI restriction site at SV40 nucleotide 358 and extending to the *Bst*NI site at SV40 nucleotide 232 (2, 8). The results of this analysis (Fig. 4) show that the Ad2MLP was equally active in pSVAdL- and pSVAdLa-transfected

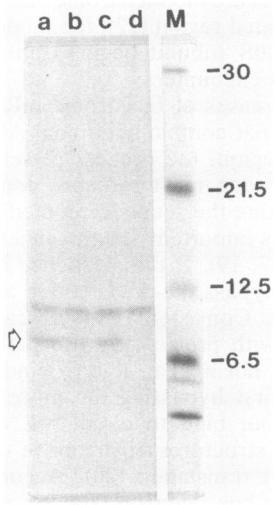

FIG. 2. Production of agnoprotein in pSVAdL-transfected cells. Immunoprecipitation of agnoprotein extracted from BSC-1 cells transfected with pSV3 (lane a), pSVAdE (lane b), pSVAdL (lane c), or no DNA (lane d). At 48 h posttransfection, 60-mm plates of BSC-1 cells were labeled with [^{14}C]leucine (344 mCi/mmol) at 17.5 μCi/ml for 2 h. Cells were collected and lysed in 50 mM Tris (pH 7.5)–150 mM NaCl–1% Triton X-100–1% sodium deoxycholate–0.1% sodium dodecyl sulfate–250 μg of phenylmethylsulfonyl fluoride per ml. After centrifugation at 48,000 × *g* for 1 h, half of each sample was immunoprecipitated with 2 μl of agnoprotein antisera (25) at 0°C. After 1.5 h, protein-antibody complexes were isolated as previously described (4) with the following modification: the staph A-protein complex was washed with 20 mM Tris (pH 7.5)–150 mM NaCl–10 mM EDTA–0.05% Nonidet P-40. Samples were resolved on a 20% polyacrylamide gel (21). Lane M, protein size markers; sizes (in kilodaltons) are shown at the right; arrow, position of agnoprotein.

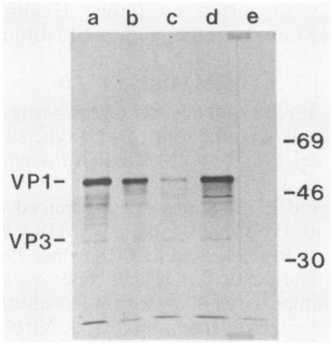

FIG. 3. Production of VPI by pSVAdLa. Immunoprecipitation of SV40 capsid proteins from BSC-1 cells transfected with pSVRI (lane a), pSVAdE (lane b), pSVAdL (lane c), pSVAdLa (lane d), or no DNA (lane e) and labeled with [^{35}S]methionine. Cells were labeled, and proteins were immunoprecipitated as described previously (8). Immunoprecipitates were washed as described in the legend to Fig. 2.

cells. S1 analysis was also performed to determine whether pSVAdL and pSVAdLa produced the same quantities of 16S and 19S late mRNA. A double-stranded DNA probe 5′ end labeled at an *Eco*RI site downstream from the late splice

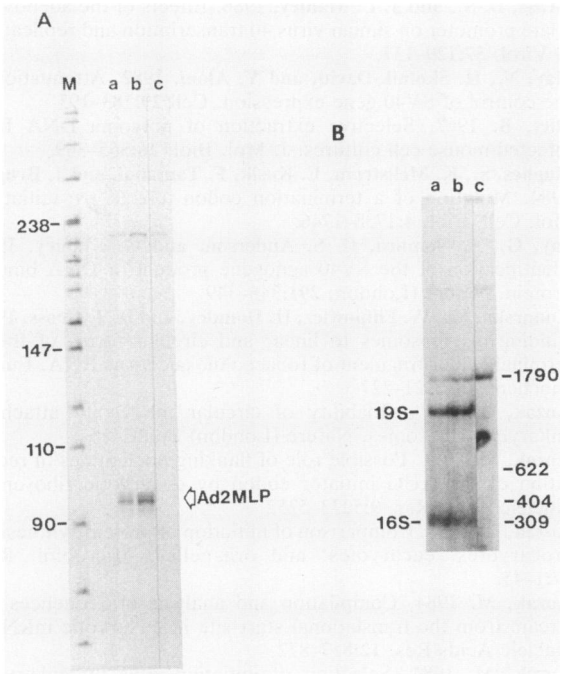

FIG. 4. (A) Expression of the Ad2MLP in pSVAdLa. S1 nuclease digestion of RNA isolated from BSC-1 cells (8) transfected with pSVAdL (lane a), pSVAdLa (lane b), or no DNA (lane c) after hybridization with a single-stranded *Bst*NI fragment covering SV40 nucleotides 232 to 358 and labeled at the 5′ end (nucleotide 358) (8). The resulting fragments were resolved on an 8% polyacrylamide sequencing-type gel (24). Lane M, DNA size markers; sizes (in nucleotides) are shown at the left. (B) Accumulation of late 19S and 16S mRNAs in pSVAdLa-transfected cells. S1 nuclease digestion of cytoplasmic RNA isolated from BSC-1 cells transfected with pSVAdL (lanes a), pSVAdLa (lanes b), or no DNA (lanes c) after hybridization with a *Bgl*I-*Eco*RI wild-type SV40 probe 5′ end labeled at the *Eco*RI site as done previously (8). S1-protected fragments were denatured with glyoxal and analyzed by electrophoresis on a 1.2% agarose gel as described previously (23).

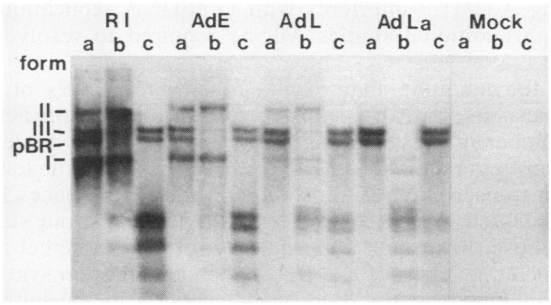

FIG. 5. Replication of viral DNAs. Southern blot analysis of low-molecular-weight DNA isolated from BSC-1 cells transfected with pSVRI (RI), pSVAdE (AdE), pSVAdL (AdL), pSVAdLa (AdLa), and no RNA (Mock). Low-molecular-weight DNA was isolated, and Southern blotting was performed as described previously (8, 30). Samples (5% of total isolate) were treated with no restriction enzyme (lanes a), *Dpn*I (lanes b), or *Mbo*I (lanes c) before electrophoresis. Electrophoresis and hybridization to a nick-translated ^{32}P-labeled pSVAdL probe were carried out as described previously (8).

acceptor sites was hybridized to cytoplasmic RNA isolated at 48 h posttransfection. The results (Fig. 4b) indicate that there is no detectable difference between pSVAdL and pSVAdLa with respect to their production of 16S and 19S mRNAs.

We showed previously that pSVAdL produced a much lower yield of virus than did either wild-type or pSVAdE, and we speculated that the lack of VP1 production might be the cause of this low titer (8). To test this possibility, BSC-1 cells were transfected as described above and incubated in 2% fetal bovine serum for 10 days. Lysates from these cells were used to infect BSC-1 cells, and virus titers were determined by plaque assays. The results indicate that, despite the ability of pSVAdLa to produce wild-type levels of VP1, the virus titer was still extremely low. The titers of pSVAdL and pSVAdLa were both reduced by a factor of approximately 50 compared with the titer of the wild type, a result indicating that very low levels of VP1 cannot solely account for the low titer of virus produced in pSVAdL-transfected cells.

In addition, it was previously determined that viral-DNA replication was significantly reduced in pSVAdL-transfected BSC-1, CV-1, and COS-1 cells when compared with replication in wild-type transfected cells (8). This result was surprising because the Ad2MLP was not inserted into SV40 viral sequences known to affect either T-antigen synthesis or viral-DNA replication. In light of the above result showing that low VP1 levels are not sufficient to explain the low titer of virus produced in pSVAdL-transfected cells, an investigation into the ability of pSVAdLa viral DNA to replicate was pursued to determine whether there was a better correlation between viability and viral-DNA replication. Low-molecular-weight DNA was isolated from BSC-1-transfected cells at 48 h posttransfection (10) and was separated on a 1% agarose gel after restriction endonuclease digestion with *Dpn*I, *Mbo*I, or no restriction enzyme. The DNA was then transferred to nitrocellulose and hybridized with pSVAdL nick-translated probe. The results (Fig. 5) indicate that replication of both pSVAdL and pSVAdLa viral sequences in BSC-1 cells was significantly reduced with respect to wild type and suggest that this defect accounts at least in part for the reduced viral yields in both pSVAdL- and pSVAdLa-transfected cells. We speculate that transcription from the

strong Ad2MLP interferes with viral-DNA replication, although additional studies will be required to resolve this issue.

Is the mutation that results in elevated levels of VP1 synthesis in pSVAdLa-transfected cells *cis* or *trans* acting? A number of previous observations have led to models of agnoprotein regulation of late gene expression at the level of either transcription or translation (e.g., see references 1 and 9; discussed in references 3 and 28). However, our studies and those of Resnick and Shenk (28) failed to detect alterations in the levels of late RNA when agnoprotein synthesis was inhibited by the T-to-A transversion in the initiation codon, thus arguing against a role for the agnoprotein in control of late transcription. Likewise, the postulated role of agnoprotein as an activator of VP1 translation (9) is not supported by these same studies. Although our results would be consistent with agnoprotein functioning as a repressor of VP1 translation, previous studies (reference 28 and references therein) argue strongly against this possibility. Thus, it is likely that the elevated levels of VP1 synthesis detected in pSVAdLa-transfected cells result from a *cis*-acting effect of the point mutation in the agnoprotein initiation codon.

We believe that our findings support a scanning model of translation (16) and suggest that the level of VP1 synthesis was restored to normal because more 40S ribosomal subunits were able to reach the VP1 AUG initiation codon. Conversely, our experiments suggest that in pSVAdL-transfected cells, reinitiation of translation after agnoprotein synthesis was very inefficient. This finding contrasts with the situation in wild-type infected cells, in which high levels of both VP1 and agnoprotein are synthesized, and thus suggests that the 33 nucleotides of the adenovirus late leader significantly reduce the ability of 16S mRNA to function bicistronically.

What is the basis for this effect on translation? Perhaps the adenoviral leader sequence causes the scanning 40S ribosome to pause shortly before it encounters the agnoprotein AUG codon, making the ribosome more likely to detect this initiation codon and in turn allowing fewer 40S ribosomal subunits to reach the AUG initiation codon for VP1. Within the 33 nucleotides of the adenovirus type 2 sequence is a 9-nucleotide sequence complementary to the 3′ end of the 18S rRNA (31). It has been proposed that this sequence might be able to facilitate initiation of translation in much the same manner that the Shine-Dalgarno sequence facilitates initiation of translation in procaryotes (29). In any event, our experiments suggest that VP1 synthesis occurs as a result of ribosomes bypassing the agnoprotein initiation codon rather than by reinitiation of protein synthesis.

pSVAdL had been shown previously to produce only low titers of virus when compared with wild type. We speculated that this was caused by the severely limited levels of VP1 in pSVAdL-transfected cells. Our experiments with pSVAdLa show that this hypothesis is not correct. Since others have shown that the absence of agnoprotein in otherwise wild-type SV40-infected BSC-1 cells had no effect on viral viability (28) and since reduced VP1 synthesis is not the cause of decreased viability in pSVAdLa-transfected cells, lack of VP1 synthesis cannot be the cause of the decreased viral viability observed in pSVAdL-transfected cells.

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